3A ASSEMBLY NUMBERS

SECTION 0: INTRO

The purpose of this document is to calculate theoretical amounts of materials required for successful restriction digest and ligation. It is ideally used as pre-lab work, to ensure that reactions are theoretically successful.

SECTION 1: RESTRICTION DIGEST

(a) Restriction digest dilutes the DNA concentration

When you mix the mini-prepped plasmid solution with the enzymes, buffer and water for restriction digest, you are effectively diluting the DNA concentration. Assume that the DNA concentration after mini-prep is 100ng/µL, a typical restriction digest with a total reaction volume of 50µL may look like[1]:

$$10\ \mu L\ \text{plasmid solution} + 5\ \mu L\ \text{buffer} + 2\ \mu L\ \text{enzymes} + 33\ \mu L\ \text{water} = 50\mu L$$

The DNA concentration after restriction digest would be:

$$\left(\frac{100\ \text{ng DNA}}{1\ \mu L}\right)\left(\frac{10\ \mu L\ \text{DNA volume}}{50\ \mu L\ \text{total volume}}\right) = \frac{20\ \text{ng DNA}}{1\ \mu L}$$

$$(Original\ DNA\ concentration)\left(\frac{\text{DNA volume}}{\text{Total volume}}\right) = Dilute\ DNA\ concentration$$

(b) Running a gel after restriction digest requires 20ng

To get a result on a gel, the sample must contain at least 20ng of the smallest fragment of DNA you want to see. Depending on the length of the parts you might need to increase the volume of the digest, the DNA concentration, or both.

> If you're digesting a 3kb plasmid and the part you're cutting out is 30bp, there must be at least 20ng of the 30bp part in the gel. Ideally after restriction digest, you have a 1:1 ratio of 30bp pieces and 2970bp pieces. Assume that the DNA concentration after restriction digest is 20ng/µL.
>
> $$\left(\frac{30\ \text{bp part}}{3000\ \text{bp total}}\right)\left(\frac{20\ \text{ng}}{1\ \mu L}\right) = \frac{0.2\ \text{ng of part}}{1\ \mu L}$$
>
> If we began with a 1µL sample, you would have 0.2ng of your 30bp part and 99.8ng of your 2970bp part, but a 1:1 ratio of 30p part to 2970bp part

Then to get 20ng, we could use 100µL for the gel.

$$\left(\frac{0.2\ \text{ng of part}}{1\ \mu L}\right)(100\mu L) = 20\text{ng of part}$$

There are two issues with this: (1) Our restriction digest volume was only 50µL (2) a gel electrophoresis well cannot hold that much solution. Even if we increased the volume of the digest to 100µL, we still have too much solution to run the gel electrophoresis. Thus we must ensure that the DNA concentration prior to digestion is the appropriate amount.

[1]NEB Protocol. The buffer volume should not exceed 10%. The buffer contains glycerol, which helps with preservation. Too much glycerol will lower the precision and specificity of the enzymes, causing them to cut at the wrong sites (star activity).

(c) Ensuring the DNA concentration prior to digestion is enough for gel

Let's assume we only want to run 10μL on the gel. If we were following NEB standard digestion protocol (1:5 DNA:Reaction volume), let x = starting concentration of DNA prior to restriction digest to be:

$$(x)\left(\frac{10\ \mu\text{L DNA volume}}{50\ \mu\text{L total volume}}\right)\left(\frac{30\ \text{bp part}}{3000\ \text{bp total}}\right)(10\mu\text{L}) = 20\text{ng}\ of\ part$$

Solving for x.

$$x = \frac{5\times 20ng}{10\mu\text{L}\times\frac{30\ \text{bp}}{3000\ \text{bp}}} = \frac{1000\ \text{ng DNA}}{1\ \mu\text{L}}$$

Starting with 1000ng DNA per μL is an unreasonable concentration. This makes sense though, if we wanted 10X less volume, we would need to start with 10X higher concentration. Thus we need to balance starting concentration with digest dilution and gel loading volume.

(d) Adjusting digest dilution

NEB protocol suggests certain digest solution ratios in order to maximize efficiency. One could increase the amount of plasmid solution and decrease the amount of water. Ideally 1μg of DNA is used for digestion, since by definition, 1 unit of restriction enzyme will completely digest 1μg of DNA in 50μL in 60 minutes. However we could increase the DNA amount and also increase the incubation time.

(e) A complete equation, in variable form

$$(\text{Initial concentration of DNA})(\text{Digest dilution})\left(\frac{\text{Part size}}{\text{Total plasmid}}\right)(\text{gel loading volume}) = 20\text{ng of part DNA}$$

$$\frac{20\text{ng}}{(\text{Initial concentration of DNA})(\text{Digest dilution})\left(\frac{\text{Part size}}{\text{Total plasmid}}\right)} = \text{Minimum gel loading volume}$$

SECTION 2: LIGATION

(a) Introduction to ratio amounts

Ligation is focused on obtaining amounts of DNA that are relative (ratios). For every 1 linearized backbone, you want X digested fragments, where X will likely be 1, 3, or 7.

(b) How much solution should we use in ligation?

Our goal is to determine how much of solution A (part) we should use and how much of solution B (backbone) we should use in a ligation. We can think of it as a 3 step process

1. Determine of weight of DNA we want to use
2. Determine the concentration of DNA you have
3. Determine the volume of DNA we want to use

(c) Determining the amount of insert DNA we want to use

$$\left(\frac{\text{size of insert (bp)}}{\text{size of vector (bp)}}\right)(\text{ng of vector})\left(\text{molar ratio of}\frac{\text{insert}}{\text{vector}}\right) = \text{ng of insert}$$

iGEM Protocol calls for 25ng of vector, so let's substitute that number. We should use however much of solution B (backbone) to get 25ng of DNA. The amount of insert DNA we should use then:

$$\left(\frac{\text{size of insert (bp)}}{\text{size of vector (bp)}}\right)(25\text{ng})\left(\text{molar ratio of}\frac{\text{insert}}{\text{vector}}\right) = \text{ng of insert}$$

(d) Determining the concentration of DNA you have

Nanodrop results after restriction-digest are unreliable since the solution is diluted with various enzymes, buffers, and other reaction materials. Instead, we should determine how much restriction digest diluted pure plasmid concentration.

$$[\text{Initial DNA}]\left(\frac{\text{DNA volume}}{\text{Total volume}}\right)\left(\frac{\text{Insert size}}{\text{Total plasmid}}\right) = [\text{Dilute insert DNA}]$$

(e) Determining the volume of DNA we want to use

$$\frac{\text{weight (ng)}}{\text{concentration }(\frac{\text{ng}}{\mu\text{L}})} = \text{volume }(\mu\text{L})$$

$$\frac{\left(\frac{\text{size of insert (bp)}}{\text{size of vector (bp)}}\right)(25\text{ng})\left(\text{molar ratio of}\frac{\text{insert}}{\text{vector}}\right)}{[\text{Initial DNA}]\left(\frac{\text{DNA volume}}{\text{Total volume}}\right)\left(\frac{\text{Part size}}{\text{Total plasmid}}\right)} = \text{volume insert DNA}$$

(f) An example: Simulating a 3A assembly with the iGEM protocol

- Ampicillin backbone (pSB1A2): 25ng in 50.0 ng/µL. 2079bp
- Part A is a promoter/RBS (K081005): 20.0 ng/µL after digest. 58bp
- Part B is a chromoprotein (K1033910): 20.0 ng/µL after digest. 714bp
- We want a 3:1 ratio of insert to backbone

How much of Part A do we need?

$$\frac{58\text{ bp}}{2079\text{ bp}} \times \frac{3\text{ mol}}{1\text{ mol}} \times \frac{25\text{ng}}{20\ (\frac{\text{ng}}{\mu\text{L}})} = 0.1\ \mu\text{L}$$

What about Part B?

$$\frac{714\text{ bp}}{2079\text{ bp}} \times \frac{3\text{ mol}}{1\text{ mol}} \times \frac{25\text{ng}}{20\ (\frac{\text{ng}}{\mu\text{L}})} = 1.29\ \mu\text{L}$$

Just for completion, how much backbone do we need?

$$\frac{25\text{ng}}{50\ (\frac{\text{ng}}{\mu\text{L}})} = 0.5\ \mu\text{L}$$

The good thing is, iGEM protocol calls for 2µL of each solution anyways. To get a higher yield, we could add proportionally more of the part B solution, but it is not necessary. So long as we have *at least* 0.1µL of part A and 1.29µL of part B, the ligation should work.

Continuing with our ligation, we would want a total reaction volume of 10µL. We'll use 1µL of T4 Ligase buffer and 0.5µL of T4 Ligase. The ___ is for water.

$$0.1\ \mu\text{L} + 1.29\ \mu\text{L} + 0.5\ \mu\text{L} + 1\mu\text{L} + \_\_\ \mu\text{L} = 10\ \mu\text{L}$$

How much of the backbone is being used? (in ng)	25
How big is the linearized backbone? (in BP)	2079
How big is Part 1, including its backbone? (in BP)	2128
How big is Part 1? (Just the part, in BP)	58
How big is Part 2, including its backbone? (in BP)	2784
How big is Part 2? (Just the part, in BP)	693
How concentrated is the linear backbone? (in ng/µL)	50
How concentrated is Part 1? (in ng/µL)	20
How concentrated is Part 2? (in ng/µL)	20
What is the desired mole ratio of parts to backbone?	3
Amount of backbone you should use (in µL)	0.5
Amount of Part 1 to use for ligation (in µL)	3.83838
Amount of Part 2 to use for ligation (in µL)	5.02165

Table for ligation calculations